Life on a beach for island lizards: phenotypic divergence in the face of gene flow

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Abstract

Morphological divergence under gene flow was investigated in the wall lizard *Teira dugesii* from the Atlantic island of Madeira island. Lizards (n=334) were sampled using a matched pairs design at four distinct coastal localities. Matched pairs comprised adjacent (<1 km) grey shingle beach and inland sites. Luminances of specific dorsal areas were recorded for each RGB channel from digital photographs taken in the field. Lizards were found to be significantly darker at beach sites than inland sites. Geometric morphometric analyses using 35 landmarks placed on dorsal photographs of the head revealed significant divergence between beach/inland habitats: wider snouts were found at beach sites. Genotyping-by-sequencing of 93 individuals provided 19311 cross-genomic SNPs. A spatial principal components analysis showed significant genomic divergence across the four sampled localities and within these localities. However, there was no evidence that beach and inland populations formed distinct lineages. Patterns of genomic divergence were compared with those generated from simulations under three models. Primary findings were repeated across all four localities. The model of divergence without gene flow was rejected, while the most strongly supported model incorporating two periods of gene flow: an early period of lower gene flow followed by a period of higher gene flow. Gene flow from inland to beach was greater than that in the opposite direction. This study demonstrates ecologically significant morphological divergence in the face of gene flow and adds to understanding of how divergence and speciation may occur within small islands.

Title Page

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Running title: Phenotypic divergence with gene flow

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Morphological divergence under gene flow was investigated in the wall lizard *Teira dugesii* from the Atlantic island of Madeira island. Lizards (n =334) were sampled using a matched pairs design at four distinct coastal localities. Matched pairs comprised adjacent (<1 km) grey shingle beach and inland sites. Luminances of specific dorsal areas were recorded for each RGB channel from digital photographs taken in the field. Lizards were found to be significantly darker at beach sites than inland sites. Geometric morphometric analyses using 35 landmarks placed on dorsal photographs of the head revealed significant divergence between beach/inland

habitats: wider snouts were found at beach sites. Genotyping-by-sequencing of 93 individuals provided 19311 cross-genomic SNPs. A spatial principal components analysis showed significant genomic divergence across the four sampled localities and within these localities. However, there was no evidence that beach and inland populations formed distinct lineages. Patterns of genomic divergence were compared with those generated from simulations under three models. Primary findings were repeated across all four localities. The model of divergence without gene flow was rejected, while the most strongly supported model incorporating two periods of gene flow: an early period of lower gene flow followed by a period of higher gene flow. Gene flow from inland to beach was greater than that in the opposite direction. This study demonstrates ecologically significant morphological divergence in the face of gene flow and adds to understanding of how divergence and speciation may occur within small islands.

Keywords

Evolution; colour; genomics; morphology; selection; speciation.

Introduction

Gene flow impedes divergence between populations by reducing differences in allele frequencies and facilitating the disruption of associations across loci. Nonetheless, detailed population genomic studies of some model organisms, including sticklebacks (Bay et al., 2017) periwinkle snails (Johannesson et al., 2017) and stick insects (Riesch et al., 2017), have shown how divergence and speciation can occur in the presence of gene flow. One scenario is the existence of strong divergent selection across different environments. In principle, divergence could accumulate around loci under selection while neutral loci will be homogenized by gene flow (Feder & Nosil, 2010). There is empirical evidence that neutral gene flow continues following colonization of a novel environment (e.g., Rosenblum et al., 2007). However, it is also possible that gene flow could be reduced at all loci mediated by, for example, selection against migration when this leads to lower fitness in the new environment (Nosil et al., 2008; Orsini et al., 2013; Sexton et al., 2014). The number of clear examples is relatively small and identification of new model organisms is needed to obtain greater insights into the population genomics of divergence between environments.

Small oceanic islands have provided excellent models for studies of divergence and speciation. Many island species occupy an unusually wide variety of environments and provide opportunities for examining divergence and gene flow between habitats. To date there do not seem to be any clear examples of within-island adaptive divergence without interruption of gene flow. Instead, many studies have shown that population divergence within islands has been mediated by either historical or current interruptions to gene flow (Roderick & Gillespie, 1998; Juan et al., 2000; Brown et al., 2006; O'Connell et al., 2021), with volcanic events such as debris avalanches and major lava flows often being implicated (Malhotra & Thorpe, 2000; Brown et al., 2006; Brown et al., 2017). In other cases, strong correlations with environmental gradients and an obvious ecological significance of the trait(s) studied seem to support the hypothesis of within-island adaptive divergence between habitats (e.g., Thorpe & Brown, 1989; Brown et al., 1991; Malhotra & Thorpe, 1991). However detailed molecular analyses have generally indicated that this was accompanied by historical interruptions to gene flow (e.g., Gübitz et al., 2005; Suárez et al., 2014).

A better understanding of how within-island divergence originates may also be important in explaining how island communities develop. Adaptive responses to different microhabitats seem to partially explain the existence of sets of species comprising distinct ecomorphs within some islands (e.g., Losos, 2009; Mahler et al., 2010) although, as described above, interruption of gene flow by spatial isolation is also likely to have been important (Wang et al., 2013). Population-level studies within a species from a single island could establish whether population isolation is a prerequisite for within-island evolution.

Robust analyses of gene flow have followed the development of methods based on the coalescent (e.g., Beerli & Felsenstein 1999; Hey & Neilsen, 2004; Hey, 2006). More recent developments have provided coalescent approaches that are highly suitable for analyses of genomic data (Excoffier et al., 2013). These methods allow assessment of different gene flow scenarios within a historical context which can be much more revealing than instantaneous estimates.

This study first aimed to test for morphological divergence in a lizard between several pairs of similar adjacent habitats: parallel patterns of divergence at different locations can substantiate the hypothesis of divergent selection (Butlin et al., 2014). Next, we aimed to test models of gene flow between matched pairs of divergent environments to establish whether potential ecomorphs had arisen with, or without, genetic isolation.

Methods

Study species

The native lizard *Teira dugesii* is endemic to the Madeira archipelago in the Atlantic and is highly abundant across most habitats in the island of Madeira (maximum elevation 1862m a.s.l, surface area 742 km^2) from sea-level to the highest peaks. It shows some environment-correlated patterns of morphological variation but, unlike some other oceanic island lizards these are quite weak (Báez & Brown, 1997) and there is little evidence of strong within-island phylogeographical patterns (Brehm et al., 2003). This study builds on previous work that described a population from an intertidal zone on a grey shingle beach in south-east Madeira, i.e., Caniço (Davenport & Dellinger, 1995). While the finding of a lizard that inhabits the seashore was quite unusual by itself, this population also displayed morphological characteristics that appeared to be adaptive, such as darker skin pigmentation (Davenport & Dellinger, 1995).

Sampling

A matched pairs design was used with grey shingle beach habitats (B) and adjacent inland habitats (I) being identified at localities from four different parts of the island (labelled 1-4, see Fig. 1 and Supplementary Table 1). Locality 1 (Caniço) was selected because it corresponded to the area described by Davenport & Dellinger (1995). Localities 2 (Porto da Cruz), 3 (Paul do Mar) and 4 (São Vicente) were selected because they contained similar B/I habitats and were quite distant (range: 13-39 km) from locality 1. Distances between habitats within localities were always less than 1km, ranging from approximately 0.2km between 4-I and 4-B, to 0.8km between 2-I and 2-B.

Lizards (216 males, 118 females) were trapped at each locality/habitat using upright plastic containers baited with fresh tomato. Sample sizes were similar for each of the eight sites (range: 35-46 individuals). All individuals were photographed (described below) with tail-tips also being removed from 93 individuals (9-14 per site) and stored in DNA/RNA Shield (Zymo Research). Sampling was authorised by the Regional Madeiran Government (Fieldwork/capture licence 10/IFCN/2018 - FAU MAD, issued on 04/12/18).

Lizard dorsal luminance and site substrate

The dorsa of all lizards were photographed using a Nikon D3300 camera with a zoom lens set at a focal length of 140mm. Photographs were taken against the same background and included a standard 24-patch colour reference target (X-Rite ColorChecker Passport Photo 2) with scale bar. From each photograph, luminances corresponding to the three RGB channels were recorded from six dorsal/head areas (see below) using the multispectral imaging plug-in Micatoolbox v1.22 (Troscianko & Stevens, 2015) within the program Imagej 1.52v (Schneider et al., 2012). Images were first normalised using known grey reflectance values for two of the X-Rite ColorChecker grey standard targets (10.17% and 59.41%). The six body characters on each lizard (four characters from the dorsal and lateral parts of the upper thorax and two characters from the head: Supplementary Figure 1) were selected to represent variation in darkness of the head and upper part of the body. Character positions were located on all specimens and then the Micatoolbox plug-in was run on all normalised images, allowing extraction of mean pixel luminances.

RGB luminance was $\log_{10}$-transformed and analysed using Discriminant Function Analysis (DFA), with individuals grouped according to the eight locality/habitat sites (sexes were analysed separately due to sexual dimorphism). Overexposed photographs (corresponding to two males) were not used (214 males and 118 females were analysed). Significance of variation between localities and habitats was tested using a two-way MANOVA (IBM SPSS Statistics ver.26).

Nine or ten standard photographs were taken at each locality/habitat to quantify differences in substrate

luminance. Each photograph was taken adjacent to a trap at which lizards were captured. Photographs contained a standard grey balance target (X-Rite ColorChecker Passport Photo 2) and a square wire quadrat ($0.25m^2$). Variation in substrate colour was assessed by comparison of means of RGB channels across quadrats, with site and locality as factors, using two-way MANOVA. Percentage vegetation cover was also recorded and compared between sites and localities.

-Fig.1

Lizard morphology

Head measurements were taken from 2D images obtained in the field using a tripod-mounted Nikon D3300 SLR camera with a 60mm Nikkor macro lens. Dorsal views of heads were photographed from a height of 30 cm with each photograph containing a scale bar. Previous laboratory testing showed that this protocol produced $< 5\%$ measurement error compared with linear measurements taken using callipers. Five of the sampled individuals were not analysed so sample sizes were 213 males and 116 females.

Variation in male and female head morphology was captured using thirty-five landmarks with the program tpsDig (Rohlf, 2015) (Supplementary Figure 2). All landmarks were recorded between the intersection of scale patterns, i.e., they were type 1 landmarks (Bookstein, 1991). The 2D landmark coordinates were used to quantify head size using the centroid size (CS), which is defined as the square root of the squared distances between each landmark and the barycentre of landmark configuration (Bookstein, 1991). Generalised Procrustes analysis was applied following the established geometric morphometric protocol (Rohlf and Slice, 1990) to standardise 2D coordinates, after translation, rotation and scaling to the unit centroid size, to obtain shape variables.

For head size, $\log_e$ transformed CS was tested for the main effects of habitat and locality and habitat-locality interaction using two-way factorial ANOVA. For shape, Procrustes ANOVA allowed testing of these main effects and their interaction (Collyer et al., 2015). DFA was used on the shape data grouped by the eight locality/habitat sample areas. Significant differences between matched sites (within localities) were tested by examining whether Mahalonobis distances deviated from zero, with probabilities obtained from randomization tests (10,000 permutations). All morphometric analyses were performed using the R package 'geomorph' v. 4.0 (Adams et al., 2021) and the program MorphoJ (Klingenberg, 2011).

Genomic data

General genomic divergence between sites was established using genotype-by-sequencing (GBS), carried out as follows by Hangzhou Lianchuan Biotechnology Co., Ltd. DNA. Total genomic DNA was extracted from the lizard tail tips. The DNA was incubated with the restriction enzymes *ApeK* I and *Pst* I (NEB, Ipswich, MA, USA) at 37 and digested DNA then recovered using magnet beads and the GBS library prepared using the NGS Fast DNA Library Prep Set (Illumina, SanDiego, CA, US). The library was purified and electrophoresed on a 2.5% agarose gel and DNA fragments of 350-450 bp were excised and diluted before paired-end sequencing on a NovaSeq 6000 platform (Illumina, SanDiego, CA, US). Quality filtration was carried out; adapters were removed using AdaptorRemoval v2 (Schubert et al., 2016) and reads with low quality eliminated using FastQC v0.10.1 (Andrews, 2010).

SNPs were called from the reads that were aligned using a GBS SNP Calling Pipeline (GBS-SNP-CROP v.4.1: Melo et al., 2016). A minimum phred score base call quality of 30 was specified. Due to the lack of a reference genome, a mock reference was created from the individual with the greatest number of reads. Following production of the variant discovery matrix containing all SNPs, variants were filtered largely using the default options except for the following: 1) alternate allele strength parameter (-altStrength) = 0.95, 2) maximum average depth of an acceptable variant (-mxAvgDepth) = 30, 3) minimum average depth of an acceptable variant (-mnAvgDepth) = 3, 4) minimum acceptable proportion of genotyped individuals to retain a SNP position (–mncall) =0.90. SNP positions that also showed major heterozygote excess were also removed (detected by deviation from Hardy-Weinberg equilibrium). We also created a subsampled ("thinned") dataset with only one SNP per tag (to remove interdependence of SNPs in close proximity).

Structuring of genomic divergence was explored using several approaches. A Discriminant Analysis of Principal Components (DAPC: Jombart, 2008) was initially used to assess divergence between sites. This involved computing a Principal Component Analysis (PCA) on all SNPs (homozygous SNPs coded 0 or 2 and heterozygous SNPs coded 1). PCs with the largest eigenvalues were then input into a DFA. The number of PCs that were retained was determined from comparisons of Root Mean Squared Error (RMSE) and Mean Successful Assignment Rate (MSAR) of individuals to groups following cross-validation (100 training sets sampled from the data).

Potentially divergent B/I selection on SNPs was examined using a two-step process. We first detected outlying SNPs using pcadapt v4.3.3 (Luu et al., 2017). Four groups were specified (k=4). At this step, outliers were defined as those with a minor allele frequency greater than 5 that had a Bonferroni-adjusted outlier p-value <0.1. An association between these SNPs and habitat variation (using allele frequencies across the eight groups) was then tested using bayenv2 (Gunther & Coop, 2013). This analysis used a covariance matrix obtained from the thinned dataset and B/I environment at each locality/habitat sample was specified using a binary variable. Bayes factors were obtained for all SNPs. Due to the stochasticity of this MCMC analysis, ten runs were carried out (with different random number seeds) with 200000 MCMC steps in each.

Spatial structuring was also investigated using spatial PCA (sPCA), as implemented in adespatial (multispati command) (Dray et al., 2018), using site latitudes and longitudes. PCA scores were used as input (no missing values and only one SNP per sequence tag). Spatial weights were obtained from a connection network with individuals within localities being defined as neighbours (using maximum and minimum distance criterion). Eigenvalue tests (9999 randomizations) were used to test for local and global structuring (see Montano and Jombart 2017).

The hypothesis that populations from B sites formed a separate lineage from the I sites was examined using Treemix (Pickrell and Pritchard, 2012). To reduce the problem of missing data, all individuals which had $>25\%$ SNPs missing were first removed, then SNPs that were missing in [?]10% of individuals were removed, and 1 SNP per tag was sampled. Support for tree nodes was obtained from 1000 bootstrap replicates.

Joint folded site frequency spectra (SFS) were used to compare three B/I scenarios of divergence at each of the four localities using the program fastsimcoal2 (ver. fsc27; Excoffier et al., 2013). The program implements a maximum likelihood approach that predicts the SFS under each scenario for comparison with the observed SFS. The scenarios that were modelled were: 1) divergence without subsequent gene flow (Nogflow), 2) divergence followed by constant gene flow (Onegflow), 3) divergence followed by two different periods of gene flow (Twogflow) to accommodate, say, higher gene flow after divergence but lower gene flow nearer to the present. All SFS were obtained from SNPs with no missing values for all individuals within the four B/I habitat pairs (the three individuals with most missing values were removed from each sampled habitat to help maximize the number of SNPs). Two sets of analyses were carried out for each B/I pair using i) datasets derived from the full SNP dataset (referred to as ALLSNP datasets and used to obtain parameter estimates), ii) datasets derived from the one SNP per sequence tag, excluding any outliers determined by the pcadapt analysis (referred to as INDSNP datasets and used for model comparisons). The greater number of SNPs in the ALLSNP datasets should provide better parameter estimation (Excoffier at el., 2013), but non-independence of SNPs may affect the robustness of likelihood-based model comparisons. Another reason for using the ALLSNP datasets for was that reasonable estimates of the number of monomorphic sites could be used, allowing a fixed mutation rate (here, 1×10^{-8} mutations/generation). The number of monomorphic sites was estimated by first calculating the reduction in the number of SNPs from the master matrix containing all potential SNPs to the final set of filtered SNPs. We then assumed that this reduction reflected the reduction from the total number of sites sequenced to the total number of sites used (i.e., those from which filtered SNPs were identified). Potential errors in inference arising from the estimation of monomorphic sites should be relatively small, because i) the number of monomorphic sites hugely exceeded the number of SNPs and was similar for all matched pairs, and ii) identification of the best gene flow model and relative comparison of parameter estimates between regions was more important than precise parameter estimation (interpretations

do not depend on absolute values).

For both ALLSNP and INDSNP analyses, estimations of the parameters that produced the greatest likelihood under each scenario were achieved using 100 optimization cycles, with $2x10^5$ coalescent simulations used to approximate the expected SFS in each cycle. This was replicated 100 times, with the replicate with the smallest deviation from the maximum observed likelihood being selected.

For the INDSNP analysis the Akaike information criterion (AIC) was compared between models. We also assessed stochastic variation in likelihood estimation by rerunning the fastsimcoal2 analyses 100 times using the parameters obtained for our best model.

Confidence intervals for the ALLSNP parameter estimates were obtained using the parametric bootstrap. For each locality, 100 SFS were generated to reflect the observed amount of genomic data structured as 300bp tags. The parameters of the best model ALLSNP (as determined from analysis of the actual dataset) for the locality analysed were used to generate these bootstrap replicates. These SFS were each analysed using the observed SFS for each run starting from the values obtained from the best run with the real data. These analyses used $1x10^5$, coalescent simulations 50 optimization cycles and 40 replicates.

Results

Colour variation

Beach individuals had lower luminance (i.e., were darker) on average than I individuals. A two-way MANOVA on $\log_{10}$-transformed R, G and B luminances of the 6 colour characters indicated that habitat, locality and habitat-locality interaction were all highly significant (habitat, Pillai's Trace=0.450, $F_{6,201}$=27.44, P<0.001; locality, Pillai's trace=0.479, $F_{18,609}$=6.43, P<0.001; interaction, Pillai's trace =0.285, $F_{18,609}$=3.55, P<0.001). The effect size for males was considerably greater for habitat (partial η^2= 0.45) than for locality (partial η^2= 0.16) and habitat-locality interaction (partial η^2= 0.10). For females, habitat, locality and the interaction were also highly significant (habitat, Pillai's trace =0.463, $F_{6,105}$=15.08, P<0.001; locality, Pillai's trace =0.399, $F_{18,321}$=2.74, P<0.001; interaction, Pillai's trace =0.338, $F_{18,321}$=2.26, P=0.003). Again, the effect size was much greater for habitat (partial η^2= 0.46) than for locality (partial η^2= 0.13) and habitat-locality interaction (partial η^2= 0.11).

DFAs revealed that most of the variation across the eight locality/habitat groups was expressed by the first two Discriminant functions (DFs). Plots of DF1/DF2 showed that, irrespective of locality, B and I individuals could generally be discriminated for both males and females (Fig. 2). Despite evidence of significant habitat-locality interaction, the general pattern showed that the direction of the difference between B and I habitats was similar at all localities.

-Fig. 2

Habitat

As expected, B and I habitats were found to differ in terms of both luminance (lower luminance at beach sites) and percentage cover (considerable cover at land sites, relative to zero cover at beach sites). A two-way MANOVA on $\log_{10}$ RGB values indicated significant effects for all terms in the model (habitat, Pillai's Trace=0.871, $F_{3,69}$=154.7, P<0.001; locality, Pillai's trace=0.270, $F_{9,213}$=2.34, P<0.001; interaction, Pillai's trace =0.442, $F_{9,213}$=4.09, P<0.001). Habitat showed a very large effect size (partial η^2= 0.87) relative to locality (partial η^2= 0.09) and the interaction (partial η^2= 0.15). The DFA of these RGB values showed almost complete separation of B and I sites (Figure 3). indicating clear differences in substrate luminance.

No vegetation cover was recorded for any of the B samples. Median vegetation cover was greater than 60% at all I habitats (see Supplementary Figure 3).

-Fig. 3

Morphology

Two-way ANOVA on head size in females revealed effects of habitat ($F_{1,108}$ = 4.298; P = 0.041; partial η^2=0.038), locality ($F_{3,116}$ = 5.36, P = 0.020; partial η^2 =0.092), and locality-habitat interaction ($F_{3,108}$ = 2.78, P = 0.044; partial η^2=0.072). Parallel results were obtained for the same analysis of head size in males (six very small specimens were outliers and removed, leaving 207 males in the final analysis): habitat ($F_{1,199}$ = 4.86, P = 0.003; partial η^2=0.024) and location ($F_{3,199}$ =4.75, P=0.003; partial η^2 =0.067) were significant and so was their interaction (F = 12.19, P<0.001; partial η^2=0.155). Although variation was significant, there were no consistent patterns of variation in mean size between habitats or between localities. Inland males from locality 2 (Supplementary Figure 4A) were the largest individuals, on average, while among females, specimens from the B site at locality 1 were the smallest (see Supplementary Figure 4B).

For head shape, the first DFs from the DFA (representing 56.9% of total variance) showed that females were divergent between B and I sites (with some overlap) at all localities (Fig 4). The same pattern was found for males, where the first two DFs represented a similar proportion of the total variance (56.5%) although the degree of overlap appeared slightly greater (Fig. 4). This direction of divergence in head shape between B and I was parallel between males and females: in general B individuals at localities had a broader snout than I individuals. The only slightly different pattern was that observed at site 2 for females only (Fig. 4F), where there was clear divergence on DF2 but not on DF1 (but the sample size for female B lizards at this locality was very small).

Divergence in generalized head shape between B and I sites at each of the four localities based on Mahalonobis distances was highly significant for both females and males (Supplementary Table 2). Divergence between the same habitats at different localities was also significant.

-Fig. 4

GBS analysis

After filtering, a total of 19311 SNPs were identified in 4135 sequence tags from 93 individuals from the eight locality-habitat groups.

Evidence of selection.- A total of 52 outlier SNPs were detected within the 19311 SNPs, after Bonferroni correction, using pcadapt. Of these, only four SNPs showed a significant association with habitat type, but none of these were located on the same tag. Three of them were significant for 7/10 or fewer bayenv replicates. Only one SNP was significant for 9/10 replicates, although the three other SNPs found on the same tag were not outliers. All 52 SNPs were removed from remaining analyses that were based on one SNP per tag.

Spatial structuring .- The DAPC provided some evidence of divergence between localities and between habitats but there was no consistent pattern of B/I divergence. Eighteen PCs were favoured as input for the DFA following cross-validation (MSAR = 56.63%, RMSE = 0.457). The first two discriminant functions (DF1 and DF2) captured most (70.0%) of the variation (Fig. 5). There was some regional separation of groups along DF1: the two south coast localities (1 and 3) appeared divergent from the two north/east coast sites 2 and 4. On DF2, I lizards from localities 2 and 3 could be clearly discriminated from B lizards from the same locality, and from B and I lizards from the other locality on the same coast.

-Fig. 5

The sPCA revealed that there was significant local structuring among neighbours within the localities (observation= 34.39, P=0.0002), as well as significant global structuring (observation= 29.02, P=0.0010).

Relationships among localities/habitats.- Treemix provided no support for the hypothesis of two main lineages, comprising respective B and I populations (Supplementary Figure 5). South coast sites 1-B and 3-B grouped together suggesting a possible relationship, but bootstrap support was very weak. Overall, the analysis grouped sites 1-I, 1-B and 3-B relative to the remaining sites but bootstrap support values were weak and there was a lack of any clear geographical pattern, suggesting little or no phylogeographic structure.

Historical gene flow.- AIC values most strongly supported the scenario of divergence followed by two different periods of gene flow at each of the localities, based on the INDSNPs dataset (exactly the same pattern was detected for the dataset using all SNPs)(Table 1). The model of divergence with no gene flow provided the worst fit to the observed SFS at all localities.

Table 1. Fastsimcoal2 comparison between models using the dataset comprising one SNP per locus (INDSNPs). Differences in likelihood between observed and simulated frequency spectra are given for all models and localities. Values in parentheses provide ΔAIC units between each model and the best model (which was TWOGFLOW at all localities). Note that even for the smallest ΔAIC (i.e., 361.4 for CONGFLOW at locality 4) the probability (with regard to no difference in minimization of information loss) for CONGLOW relative to TWOGFLOW is extremely small ($1.1x10^{-157}$).

	Locality	**Locality**	**Locality**	**Locality**
Gene flow model	**1**	**2**	**3**	**4**
TWOGFLOW	9.35 (0)	4.84 (0)	6.60 (0)	6.49 (0)
CONGFLOW	111.57 (460.7)	108.44 (692.0)	108.44 (463)	86.27 (361.4)
NOGFLOW	202.07 (871.4)	272.66 (1221.4)	179.91 (786.2)	154.67 (670.4)

The favoured model (TWOGFLOW) indicated lower migration rates per individual immediately following divergence, followed by a more recent period of higher migration rates at all four localities (see Table 2 for greater detail).

Table 2. FASTSIMCOAL2 parameter estimates and corresponding bootstrap values for the model allowing two periods of gene flow for the ALLSNP dataset. Time represents the timing of the beginning of the period (in generations before present). Beach->Inland and Inland->Beach give migration rates from one habitat to the other (more specifically, the probability of a lineage present within either the beach or inland habitat moving to the other habitat, going forwards in time). The 95% bootstrap confidence intervals for all estimates are given in parentheses.

	Recent period	*Recent period*	*Recent period*	*Ancient period*	*Ancient period*	*Ancient period*
Locality	**Time**	**Beach->Inland**	**Inland->Beach**	**Time**	**Beach->Inland**	**Inland->Beach**
Locality 1	1046 (1245, 2069)	$9.81x10^{-4}$ (<1 $x10^{-6}$, $3.13x10^{-3}$)	$1.07x10^{-3}$ ($4.26x10^{-4}$, 4.52 $x10^{-3}$)	175012 (27791, 641032)	<1 $x10^{-6}$ (<1 $x10^{-6}$, 8.37 $x10^{-4}$)	$3.58x10^{-5}$ (9.12 $x10^{-6}$,5.11 $x10^{-5}$)
Locality 2	1915 (2295,20850)	$6.47x10^{-4}$ (<1 $x10^{-6}$, $2.29x10^{-3}$)	$7.26x10^{-4}$ ($3.48x10^{-4}$,3.36x10^{-3})	246721 (33310,738041)	<1 $x10^{-6}$ (<1 $x10^{-6}$,5.99 $x10^{-4}$)	$2.55x10^{-5}$ (<1 $x10^{-6}$,2.29 $x10^{-3}$)
Locality 3	537 (320,595)	$2.34x10^{-4}$ (2.28 $x10^{-4}$, 5.37 $x10^{-4}$)	1.64 $x10^{-2}$ (1.10 $x10^{-2}$,3.23 $x10^{-2}$)	797338 (104552,854471)	<1 $x10^{-6}$ (<1 $x10^{-6}$, <1 $x10^{-6}$)	<1 $x10^{-6}$ (<1 $x10^{-6}$, <1 $x10^{-6}$)
Locality 4	1095 (107,1797)	$9.08x10^{-4}$ (4.80 $x10^{-4}$, 6.40 $x10^{-3}$)	1.01 $x10^{-3}$ (<1 $x10^{-6}$,3.92 $x10^{-3}$)	1709064 (17383, 21810456)	<1 $x10^{-6}$ (<1 $x10^{-6}$,8.32 $x10^{-4}$)	$5.32x10^{-5}$ (<1 $x10^{-6}$,1.08 $x10^{-4}$)

Discussion

This study demonstrates morphological divergence between lizards found in distinct grey shingle beach

and inland environments less than 1km apart. Most notably, this pattern is repeated at four unconnected beaches across Madeira. The direction of change between habitats for both head morphology and dorsal colour appears to be replicated at all localities and for both males and females, i.e., generally broader snout and darker dorsal coloration at beach sites, providing support for the hypothesis that divergent selection between the two environments is high enough to overcome gene flow. Similar patterns of ongoing gene flow between environments were found at each locality. The genomic data did not support the hypothesis that beach-inland divergence was due to distinct evolutionary lineages occupying the different environments.

Beach/inland morphological divergence had been reported previously at one of our localities (Caniço: Davenport & Dellinger, 1995) but our findings differ in detail. The previous study described differences in perceived darkness of the lizards and relative digit and tail length, but not in relative head width (after adjustment for body length). Hence, the variation that we found in head shape was novel and largely unexpected. Although the morphological divergence in colour and head morphology is highly significant, it is also clear that there is considerable morphological overlap between habitats for both of these sets of traits. This would largely be expected under gene flow.

Genomic analyses of models of divergence showed similarities at all matched pairs of beach and inland sites. The same gene flow scenario was supported at all four localities: initial divergence (colonisation of the beach from the inland) was proceeded by a long period of lower gene flow, prior to higher gene flow during more recent times. These findings do not rely on absolute parameter estimates, but still consistently show asymmetric gene flow with generally higher rates from inland to beach sites compared with opposing rates. This finding is quite intuitive. In contrast, the finding that historical lower gene flow is relatively lower than more recent gene flow is less intuitive. Recent gene flow estimates were generally one or more orders of magnitude higher than equivalent estimates during the ancient period of gene flow and are again substantiated by being repeated across the four study areas. A finding of higher gene flow after initial colonisation of the beach environment followed by a decrease over time due to (say) evolution of assortive mating at the ecotone (e.g., Rolán-Alvarez et al., 1999) or reduced migration between habitats would ostensibly appear more likely. There is no obvious historical scenario that explains this, although initial colonisation of beaches could potentially have created less-connected coastal demes, with lower migration rates that subsequently increased after changes in coastal topography/sea-level. Sea-level fluctuations have had an impact on coastal communities (Ludt & Rocha, 2015) with dramatic changes in habitat availability expected even during the recent 18000-6000 years BP period when ˜130m rises in sea level were evident (Lambeck, 1990).

In vertebrates, between-population divergence in colour mediated by divergent selection has been reported for several lizards from different habitats such as three species that have colonized white gypsum substrates found at White Sands in New Mexico (Rosenblum, 2006), and also different species of *Peromyscus* mice in Florida and Nebraska (Sumner, 1926; Dice, 1941). Morphological divergence has also been detected between populations of a *Tropidurus* lizard species from rock outcrops and Savannah habitats in Roraima, Brazil (Vitt et al., 1997). However, in many of these cases the geographic separation is substantially greater than that for *T. dugesii* , even though high gene flow is still inferred (Pfeifer et al., 2018). Here, the very close proximity of the distinct beach and inland populations should facilitate very high gene flow and therefore dilute the effects of selection. A non-vertebrate example shows how this is possible under very divergent selection pressures. The marine gastropod *Littorina saxatalis*differs between intertidal shoreline heights that can be as close as ˜10 m apart, although it is also likely that migration rates are much lower than those for *Teira dugesii* (Janson, 1983).

There are occasional reports of other lizards being found inhabiting the shoreline, which have included island wall lizards (e.g., Cascio & Pasta, 2006), other island squamates such as skinks (Janssen et al., 2015), *Uta* (Grismer 1994), *Microlophus* (Sepúlveda, 2014) and the well-known Galapagos marine iguana which is intertidal/subtidal (Hobson, 1965). But to our knowledge morphologically divergent intertidal populations have not been described. Future studies will be useful in determining whether the same mutations underpin divergence at different localities or not. For example, it is feasible that some or all of the described divergence is due to changes in allele frequency at the same loci. Whatever the genetic basis, there is large variation

between mean estimates of initial timing of divergence which suggests that beach colonization may have occurred at different times across the four localities despite the degree of beach-inland morphological variation being similar. This further supports the idea that morphological divergence originates from natural selection, but is similarly tempered by ongoing gene flow. Several recent studies of morphological differences between rural and urban environments (e.g., Balakrishna et al., 2021; Falvey et al., 2020; Marnocha et al., 2011) suggest that the estimated divergence times here are very long relative to the short times required for morphological divergence to become detectable.

How might divergent selection operate in the different habitats? Clearly, lower dorsal luminance (darker lizards) is expected to enhance crypsis on the darker shingle beaches, as originally noted by Davenport and Dellinger (1995) and a brown/green coloration appears to be a better match to inland habitats. During fieldwork, Kestrels (*Falco tinnunculus*) were seen nesting and hunting by the coast. No predation was directly observed, but bird predation on this species is thought to be quite intense (Crisp et al., 1979; Rocha et al., 2010; Völkl & Brandl, 1988). There are several possible explanations of divergent selection on head morphology. One hypothesis is that it could be associated with diet, as proposed by Davenport & Dellinger (1995), for example. These authors showed that intertidal lizards fed on the large marine isopod, *Ligia italicus* , that was not available to inland individuals. Head size is known to greatly affect bite force and prey handling ability in lacertid lizards (e.g., Verwaijen et al., 2002) and so broader snouts could provide advantages for large/tough prey in the intertidal zone. Wider head width is also thought to frequently co-evolve with sit-and-wait predation relative to wider-foraging in lizards (McBrayer & Corbin, 2007) so it is possible that head divergence is linked to behavioural differences. Alternatively, some studies have shown that intertidal lizards might eliminate high sodium and chloride loads though nasal salt glands (e.g., Hillman and Pough, 1976) and this could be associated with the wider snout tips in beach individuals.

In general, this study shows that within-island divergence (and potentially speciation) can originate from differences between habitats alone, without requiring significant interruption to gene flow. Island topographies, particularly elevations, can lead to extremely heterogeneous environments and this variation is often correlated to within-island variation in lizard morphology (e.g., Thorpe and Brown, 1989; Brown et al., 1991; Malhotra & Thorpe, 1991). We show that environmental differences between beach and inland habitats may have a much greater impact on morphology than do other quite substantial environmental differences across inland sites (Báez & Brown, 1997). Overall, we suggest that substantial within-island morphological divergence is most likely to arise when there is either i) divergent selection that is strong enough to overcome gene flow and may arise following colonization of a novel environment (such as the shoreline) as shown here, or ii) historical population fragmentation that has impeded gene flow, as shown by previous studies.

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Data Accessibility

All data presented in this manuscript will be archived in Dryad before publication

Benefit-Sharing

Our group is committed to international scientific partnerships, as well as institutional capacity building. A research collaboration was developed with scientists of three different nationalities from institutions in two different countries in order to complete this project. One of the co-authors (JT) is a junior pre-doctoral researcher. The results of this research will be shared through this publication and a summary report to the grant-awarding body – the British Ecological Society. The study addresses an endemic insular species which are increasingly under threat from invasive species. The benefits of the work will accrue from the sharing of our data on public databases as described above.

Author Contributions

Design: RPB, CM. Data recording/genomic sequencing: RPB, CM, YJ, JT. Data analyses: RPB, CM. Manuscript: RPB, CM.